蝶と蛾 *Tyô to Ga*, 38 (4): 269－286, 1987

Species Diversity in the Island Populations of *Papilio bianor* Complex in Japan Considered from Gene Flow and Genetic Distance

Hirohiko KOMINAMI, Osamu YAMAGUCHI* and Kaoru SUMIYOSHI

Department of Science Education, Department of Biology* and Department of Applied Physiology,
Hyogo University of Teacher Education,
Yashiro-cho, Kato-gun, Hyogo, 673-14 Japan

Abstract A total of 163 adult males of *P. bianor* complex were collected in 1986 at three localities on the mainland of Japan, on the main island of Okinawa, and on Ishigaki Island. The butterflies collected from these localities are classified taxonomically into separate subspecies and make up a *P. bianor* complex. Fluid extract of the individual male was analyzed with gel-electrophoresis at six isozyme loci. The gene flow and genetic distances between these populations were examined using the resultant allelic frequencies. Specific alleles were fixed independently, both in the alcohol dehydrogenase locus and in the acid phosphatase locus between the three populations. There was no indication of geneflow between the populations. The remaining four isozyme loci depicted similar patterns of divergence, but with less prominence. Genetic distances, calculated by the formula of NEI (1972), were 0.896 between populations on the mainland of Japan and on the main island of Okinawa, 0.737 between populations on the mainland of Japan and on Ishigaki Island, and 1.268 between populations on the main island of Okinawa and on Ishigaki Island, respectively. The magnitudes of these distances strongly suggest that the divergence observed in the island populations of *P. bianor* complex is significantly greater than that of interpopulationals within a single species. Allopatric speciation is in progress with the aid of random fixation of genes due to the limited number of individuals in island populations of *P. bianor* complex.

Introduction

Geographical isolation is an initial stage of speciation. Japanese islands located in the middle part of the northern Pacific archipelago from Kamchatka to Formosa seem to provide good experimental materials for evolution. There are more than one thousand islands, some of which provide suitable habitats to butterflies. Geographical isolation is therefore expected to have a significant effect so that some types of speciation may be observed. The swallow-tail butterfly, *Papilio bianor*, is distributed widely in the Far East, and it is found throughout the Japanese archipelago. This butterfly has a beautifully colored pattern on its wings, which attracts collectors. Geographical variations in color pattern and color tone have frequently been observed and described (cf. KAWAZOÉ and WAKABAYASHI, 1976). Numerical taxonomy has been applied to distinguish some local varieties (e.g. IZUMI *et al.*, 1984). At present, they are divided into seven subspecies, based mainly on morphological differences

*Correspondence should be addressed.

(FUJIOKA, 1975). Experiments in hybrid inviability and hybrid sterility between subspecies have been tried, but the outcome has not yet been sufficient to confirm this point because of difficulties in the breeding of larvae. However, data have gradually been accumulated (e.g. AE, 1971 and 1985; HAMA, 1976; WAKI, 1981). Recently, KAWAZOÉ and WAKABAYASHI (1976) and ICHINOSE and HORIUCHI (1985) described isolates on the Okinawa Islands and on Ishigaki Island as new distinct species derived from *P. bianor*. Taken together, these experimental results suggest that speciation of *P. bianor* complex is proceeding in the northern Pacific archipelago. In this study, an experiment was designed to detect genetical differentiation between some island populations of this species complex, and to estimate how they are genetically separated from each other. Isozyme genes were chosen in this experiment because they are considered to be effectively neutral to natural selection and therefore suitable for use in the genetic distance analysis (cf. a review of KIMURA, 1982).

Materials and Methods

Materials: Adult males of *Papilio bianor* CRAMER, 1777, were collected at the following three localities. (1) Fifty-one males were collected at Akazai, Haga-cho, Hyogo prefecture in late May, 1986. These samples represent stocks of subspecies of *P. bianor dehaanii*. (2) Fifty-two males were collected at Hanechi, Nago, on the main island of Okinawa in early April, 1986. The samples correspond to stocks of subspecies of *P. bianor ryukyuensis*. However, a recent description by KAWAZOÉ and WAKABAYASHI (1976) has classified this subspecies into the new sibling species of *P. okinawensis*. Thus, in this article the samples from the main island of Okinawa were tentatively named *P. okinawensis*. (3) Sixty males were collected at a vicinity of Mt. Bannadake on Ishigaki Island in late September, 1986. These samples correspond to stocks of subspecies of *P. bianor okinawensis* (=*P. bianor junia* JORDAN) (KAWAZOÉ and WAKABAYASHI, 1976).

As a control experiment, *Papilio maackii tutanus* FENTON, 1881, a sibling species to *P. bianor*, and two unrelated species, *Luehdorfia japonica* LEECH, 1889, and *L. puziloi inexpecta* SHELJUZHKO, 1913, were employed. Fifty-three adult males of *P. maackii* were collected at Akazai, Haga-cho, Hyogo prefecture in late May, 1986. Nineteen males of *L. japonica* were collected at Nishiwaki, Hyogo prefecture in mid April, 1986. Twenty-nine males of *L. puziloi* were sampled at Fujimicho, Nagano prefecture in mid May. These butterflies were immediately put into a deep freezer after collection and kept at −80℃. Before use, samples were dissected. The isolated testes and arrayed tissues were homogenized with a small amount of distilled water in a ceramic grinder. After a short period of centrifugation at 15000 rpm, the supernatant was utilized as sample solution.

Electrophoresis: Isozyme assays were carried out using 5% polyacrylamide gel or 1% agarose gel electrophoresis. The concentration and pH condition of gel buffer and electrode buffer is shown in Table 1. Running time was 2.0 hours with a current of 50 mA. Assayed enzymes were malate dehydrogenase (Mdh), alcohol dehydrogenase

Table 1. Buffers and components of stain solution for six enzymes used for gene analysis of *P. bianor* complex.

Enzyme	Mdh	Adh	Tox	Acph	Est-α	Est-β
Gel and electrophoresis buffer	90mM EBT buffer (pH 8.7)	90mM EBT buffer (pH 8.7)	90mM EBT buffer (pH 8.7)	13mM Phosphate buffer (pH 6.8)	13mM Phosphate buffer (pH 6.8)	13mM Phosphate buffer (pH 6.8)
Stain solution	50mM Tris-HCl buffer (pH 8.5)	50mM Tris-HCl buffer (pH 8.5)	50mM Tris-HCl buffer (pH 8.5)	Distilled water	Distilled water	Distilled water
	5mM DL-Malic acid	2.5% Isopropanol		7mM α-Naphthyl acid phosphate	2% α-Naphthyl acetate in acetone	8% β-Naphthyl acetate in acetone
	0.4mM NAD 0.3mM NBT 0.4mM PMS	0.4mM NAD 0.3mM NBT 0.4mM PMS	0.4mM NAD 0.3mM NBT 0.4mM PMS	0.005% Acetic acid 1mM $MgCl_2$ 20mM Sodium acetate 1mM Naphthanil diazo blue B	1mM Naphthanil diazo blue B	1mM Naphthanil diazo blue B

(Adh), tetrazolium oxidase (Tox), acid phosphatase (Acph), esterase-α (Est-α) and esterase-β (Est-β). The stain method followed STONE *et al.* (1968) with slight modification, which is also shown in Table 1. The first three enzymes are classified as substrate-specific enzymes, and the latter three as substrate-nonspecific ones (KOJIMA, GILLESPIE and TOBARI, 1972).

Experimental Results

1. Genetic variability at isozyme loci

Frequencies of the electrophoretic patterns detected in each population of *P. bianor* are shown in Table 2. The allelic frequencies at each isozyme locus are described below.

Malate dehydrogenase (*Mdh*): There existed three alleles of Mdh distinguishable by agarose gel electrophoresis, the fastest allele toward the cathode (named Mdh^F), the middle allele (Mdh^M) and the slowest allele (Mdh^S), in the Akazai population of *P. bianor*. Their frequencies were 0.235, 0.549, and 0.216, respectively. There were three alleles in the Ishigaki population of *P. bianor*, Mdh^F, Mdh^H and Mdh^P, whose frequencies were 0.267, 0.450, and 0.283, respectively. The mobility of Mdh^H was intermediate between Mdh^F and Mdh^M, and that of Mdh^P was intermediate between Mdh^M and Mdh^S. There were also three segregated alleles, Mdh^F, Mdh^H and Mdh^P, at the frequencies of 0.288, 0.442 and 0.269 in the Nago population of *P. bianor* (=*P. okinawensis*). Nomenclature of the alleles is in alphabetical order; i.e. the higher letter is of faster mobility. The above three populations shared the common allele Mdh^F at almost the same frequencies. In the control experiment, three alleles Mdh^F, Mdh^G and Mdh^N were observed in the Akazai population of *P. maackii*. Two alternative alleles of Mdh^R and Mdh^T were detected in the Nishiwaki population of *L. japonica*. Monomorphism fixed with Mdh^T was observed in the Fujimicho population of *L. puziloi*. These allelic frequencies are summarized in Table 3. An example of the zymogram of Mdh is shown in Figure 1. The locus of Mdh was fairly high polymorphic, however a heterozygotic zymograph had never been observed in these materials.

Table 2. Phenotypic frequencies and their deviation from Hardy-Weiberg equibrium at 6 isozyme loci in three natural populations of *Papilio bianor* complex and a population of its sibling species of *P. maackii* and two populations of its unrelated species of *Luehdorfia japonica* and *L. puziloi*.

Species Locality No. ind.	*P. bianor* AKAZAI 51			*P. bianor* ISHIGAKI 60			*P. okinawensis* NAGO 52			*P. maackii* AKAZAI 53			*L. japonica* NISHIWAKI 19			*L. puziloi* FUJIMICHO 29		
Locus	Pheno-type	Obs.	Exp.	Pheno-type	Obs.	Exp.	Pheno-type	Obs.	Exp.	Pheno-type	Obs.	Exp.	Pheno-type	Obs.	Exp.	Pheno-type	Obs.	Exp.
Mdh	F	12	12.0	F	16	16.0	F	15	15.0	F	29	29.0	R	8	8.0	T	29	29.0
	M	28	28.0	H	27	27.0	H	23	23.0	G	23	23.0	T	11	11.0			
	S	11	11.0	P	17	17.0	P	14	14.0	N	1	1.0						
Adh	FF	44	42.4	OO	60	60.0	GG	50	48.1	EE	8	13.3	OO	19	19.0	OO	29	29.0
	FS	5	4.6				OO	2	0.1	FF	1	0.0						
	SS	0	0.1				GO	0	3.8	HH	6	11.3						
	OO	2	0.1							EH	37	24.5						
	FO	0	3.6							OO	1	0.0						
	OS	0	0.2							FO	0	0.0						
										HO	0	1.0						
										EO	0	1.0						
										EF	0	1.0						
										FH	0	0.9						
Dev. from H.W. eq.	$\chi^2_{df=1}=0.35$									$\chi^2_{df=2}=11.92^{**}$								
Tox	SS	51	51.0	SS	49	49.5	SS	52	52.0	FF	6	9.6	TT	14	12.7	VV	29	29.0
				UU	0	0.5				SS	14	17.5	VV	2	0.6			
				SU	11	10.0				FS	33	25.9	TV	3	5.7			
Dev. from H.W. eq.				$\chi^2_{df=1}=0.03$						$\chi^2_{df=2}=3.99$			$\chi^2_{df=1}=0.43$					
Acph	FF	10	7.4	KK	8	4.8	QQ	52	52.0	LL	52	51.0	EE	19	19.0	DD	29	29.0
	FM	5	11.1	KP	8	10.8				OO	1	0.0						
	MM	6	4.1	PP	9	6.0				LO	0	2.0						
	FS	14	13.0	KR	10	13.6												
	MS	12	9.7	PR	12	15.2												
	SS	4	5.7	RR	13	9.6												
Dev. from H.W. eq.	$\chi^2_{df=4}=4.87$			$\chi^2_{df=4}=7.04$														
Est-α	FF	51	51.0	FF	60	60.0	SS	52	52.0	SS	53	53.0	TT	19	19.0	TT	29	29.0
Est-β	FM	5	4.5	LL	14	18.7	MM	52	52.0	MM	51	51.0	UU	19	19.0	UU	29	29.0
	MM	40	40.6	LR	39	29.6				KM	1	0.9						
	MS	5	4.5	RR	7	11.7				TM	1	0.9						
	KM	1	0.9							KK	0	0.0						
	FF	0	0.1							TT	0	0.0						
	SS	0	0.1							KT	0	0.0						
	KK	0	0.0															
	FS	0	0.2															
	FK	0	0.1															
	KS	0	0.1															
Dev. from H.W. eq.	$\chi^2_{df=1}=0.04$			$\chi^2_{df=2}=6.06^{*}$														

*Significant at 5% level.
**Significant at 1% level.

A possible explanation seems to be that the Mdh locus is sex-linked in Papilionidae butterflies (see Discussion).

Alcohol dehydrogenase (Adh): A small amount of variability was detected in this locus through agarose gel electrophoresis. There were three alleles segregated in the Akazai population of *P. bianor*. The main allele was Adh^F at a frequency of 0.912. The remaining two alleles, Adh^S and Adh^O, were at the low frequencies of 0.049 and

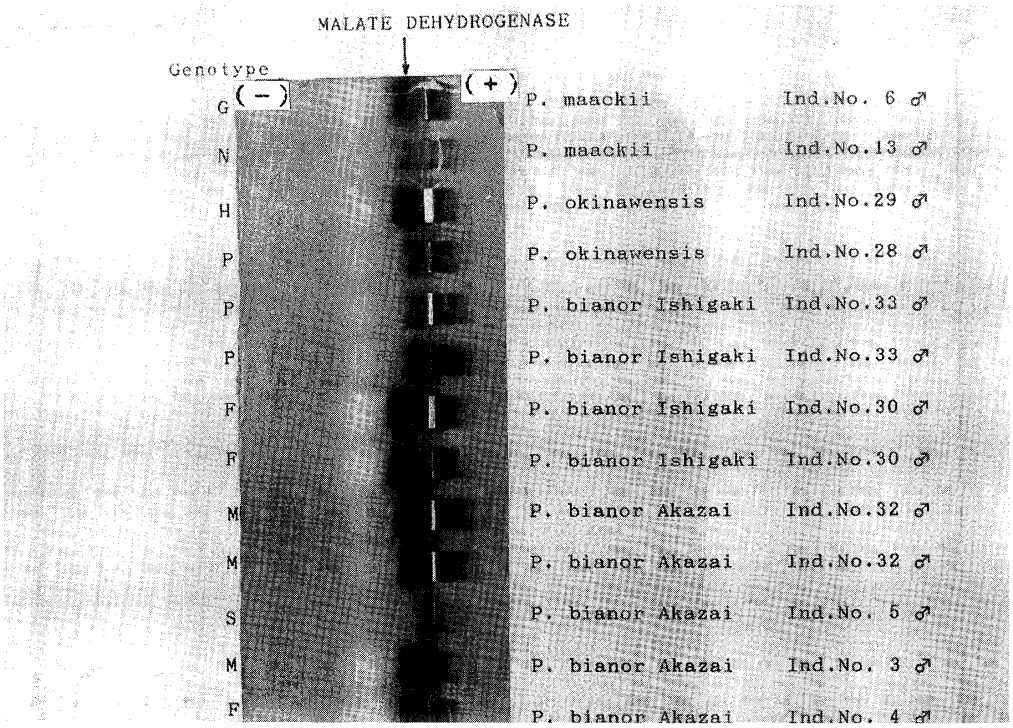

Fig. 1. An example of the zymogram of malate dehydrogenase (Mdh) in the subspecies of *Papilio bianor* complex and its related species of *P. maackii*. Isozymes of Mdh are stained in a deep blue color. White spots are the isozymes of tetrazolium oxidase (Tox). *P. okinawensis* is synonymous with *P. b. ryukyuensis* collected at Nago, Okinawa. *P. bianor* from Ishigaki is synonymous with *P. b. okinawensis* collected at Ishigakijima, Okinawa. *P. bianor* from Akazai is synonymous with *P. b. dehaanii* collected at Akazai, Hyogo. (+) and (−) stand for the directions toward anode and cathode, respectively.

0.039, respectively. The latter indicated no enzyme activity and was named null allele (Adh^0). In adult males of the Ishigaki population of *P. bianor*, no individuals showed any Adh activity at all, and were thus scored as monomorphic fixed with Adh^0. There were two alleles Adh^G and Adh^0 at the frequencies of 0.962 and 0.038, respectively, in the Nago population of *P. okinawensis*. Therefore, there was no indication of gene flow except the negligible null alleles between these three populations of *P. bianor* complex one with another. In its sibling species, *P. maackii*, a total of four alleles, Adh^E, Adh^F, Adh^H and Adh^0, were segregated at appreciable frequencies. In both of its unrelated species of *L. japonica* and *L. puziloi*, no individual males showed any Adh activity. Thus, they were scored as monomorphic fixed with Adh^0. An example of the Adh zymogram is shown in Figure 2. A heterozygote has two separable bands as in seen in the figure. Thus, the enzyme seems to be a monomer in protein structure. Allelic frequencies are also shown in Table 3.

Tetrazolium oxidase (*Tox*): This isozyme analysis was carried out basically on the same gel plate as for Mdh or Adh. The locus was rather monomorphic with the commom allele Tox^S in every population of *P. bianor* complex. Populations of both Akazai and Nago were fixed with this allele. The Ishigaki population contains an

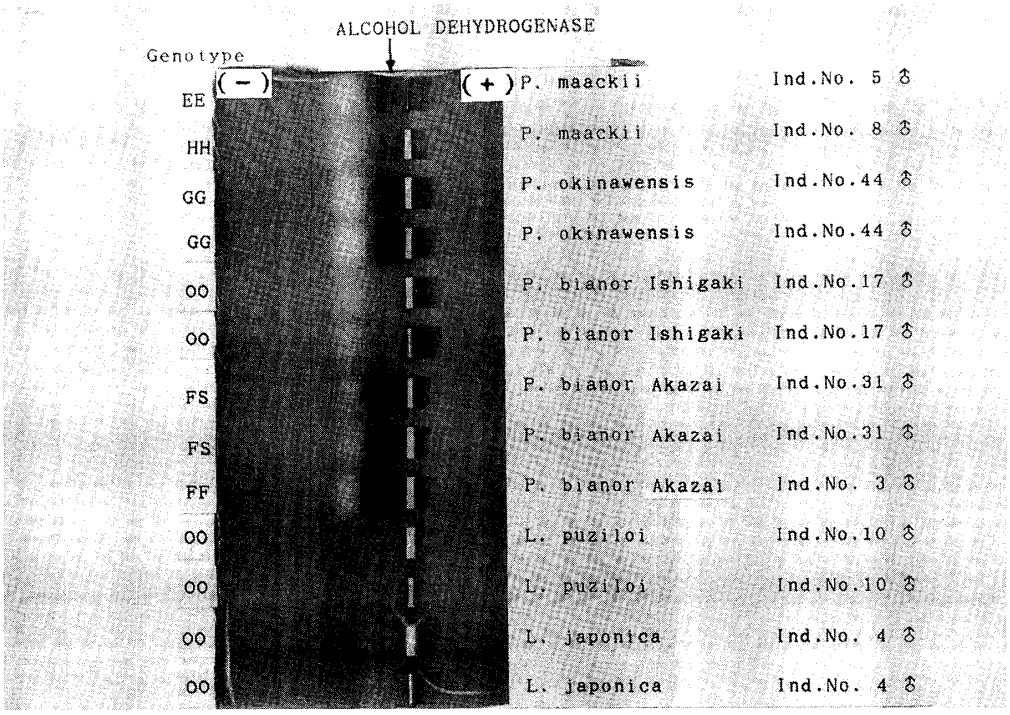

Fig. 2. An example of the zymogram of alcohol dehydrogenase (Adh) in the subspecies of *Papilio bianor* complex, in its related species of *P. maackii*, and in non-related species of *Luehdorfia japonica* and *L. puziloi*. Isozymes of Adh are stained in a deep blue color. For other explanation, see the legend of Figure 1.

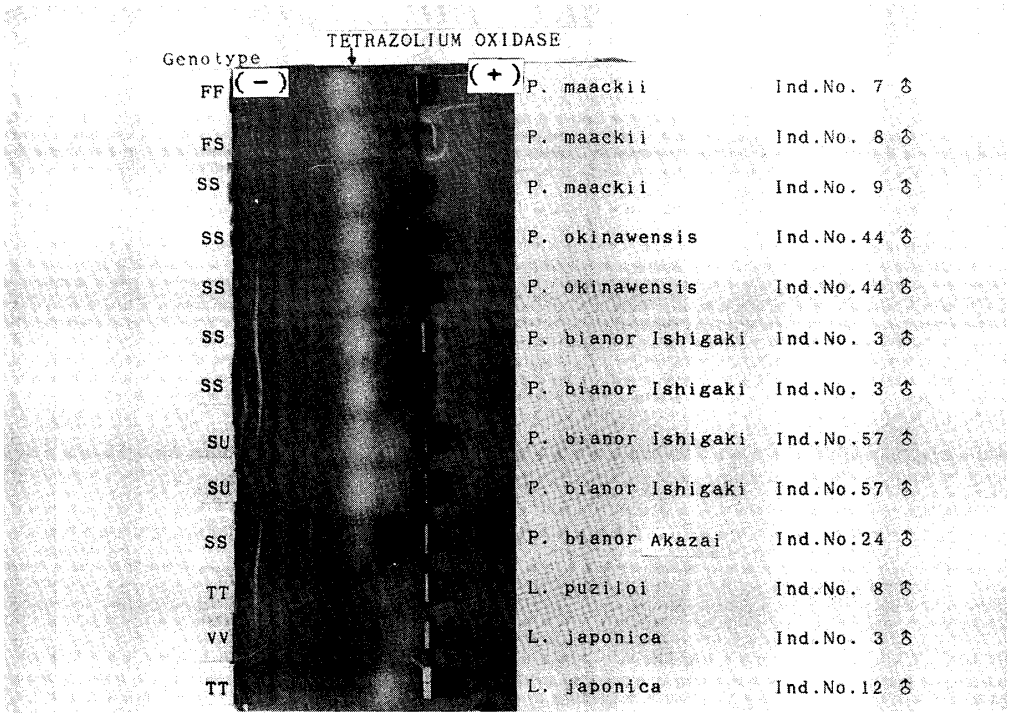

Fig. 3. An example of the zymogram of tetrazolium oxidase (Tox) in the subspecies of *Papilio bianor* complex. Isozymes of Tox are in white spots. For other explanation, see the legends of Figures land 2.

Table 3. Allelic frequencies and average heterozygosities at 6 isozyme loci in three natural populations of *Papilio bianor* complex, in a population of its sibling species of *P. maackii* and in two populations of its unrelated species of *Luehdorfia japonica* and *L. puziloi*.

Species Locality No. of genomes examined		*P. bianor* AKAZAI 102	*P. bianor* ISHIGAKI 120	*P. okinawensis* NAGO 104	*P. maackii* AKAZAI 106	*L. japonica* NISHIWAKI 38	*L. puziloi* FUJIMICHO 58
Locus	Allele						
Mdh	Mdh^F	0.235	0.267	0.288	0.547	0.0	0.0
	Mdh^G	0.0	0.0	0.0	0.434	0.0	0.0
	Mdh^H	0.0	0.450	0.442	0.0	0.0	0.0
	Mdh^M	0.549	0.0	0.0	0.0	0.0	0.0
	Mdh^N	0.0	0.0	0.0	0.019	0.0	0.0
	Mdh^P	0.0	0.283	0.269	0.0	0.0	0.0
	Mdh^R	0.0	0.0	0.0	0.0	0.421	0.0
	Mdh^S	0.216	0.0	0.0	0.0	0.0	0.0
	Mdh^T	0.0	0.0	0.0	0.0	0.579	1.000
Adh	Adh^E	0.0	0.0	0.0	0.500	0.0	0.0
	Adh^F	0.912	0.0	0.0	0.019	0.0	0.0
	Adh^G	0.0	0.0	0.962	0.0	0.0	0.0
	Adh^H	0.0	0.0	0.0	0.462	0.0	0.0
	Adh^S	0.049	0.0	0.0	0.0	0.0	0.0
	Adh^O	0.039	1.000	0.038	0.019	1.000	1.000
Tox	Tox^F	0.0	0.0	0.0	0.425	0.0	0.0
	Tox^S	1.000	0.908	1.000	0.575	0.0	0.0
	Tox^T	0.0	0.0	0.0	0.0	0.816	1.000
	Tox^U	0.0	0.092	0.0	0.0	0.0	0.0
	Tox^V	0.0	0.0	0.0	0.0	0.184	0.0
Acph	$Acph^D$	0.0	0.0	0.0	0.0	0.0	1.000
	$Acph^E$	0.0	0.0	0.0	0.0	1.000	0.0
	$Acph^F$	0.382	0.0	0.0	0.0	0.0	0.0
	$Acph^K$	0.0	0.283	0.0	0.0	0.0	0.0
	$Acph^L$	0.0	0.0	0.0	0.981	0.0	0.0
	$Acph^M$	0.284	0.0	0.0	0.0	0.0	0.0
	$Acph^P$	0.0	0.317	0.0	0.0	0.0	0.0
	$Acph^Q$	0.0	0.0	1.000	0.0	0.0	0.0
	$Acph^R$	0.0	0.400	0.0	0.0	0.0	0.0
	$Acph^S$	0.333	0.0	0.0	0.0	0.0	0.0
	$Acph^O$	0.0	0.0	0.0	0.019	0.0	0.0
Est-α	Est-α^F	1.000	1.000	0.0	0.0	0.0	0.0
	Est-α^S	0.0	0.0	1.000	1.000	0.0	0.0
	Est-α^T	0.0	0.0	0.0	0.0	1.000	1.000
Est-β	Est-β^F	0.049	0.0	0.0	0.0	0.0	0.0
	Est-β^K	0.010	0.0	0.0	0.009	0.0	0.0
	Est-β^L	0.0	0.558	0.0	0.0	0.0	0.0
	Est-β^M	0.892	0.0	1.000	0.981	0.0	0.0
	Est-β^R	0.0	0.442	0.0	0.0	0.0	0.0
	Est-β^S	0.049	0.0	0.0	0.0	0.0	0.0
	Est-β^T	0.0	0.0	0.0	0.009	0.0	0.0
	Est-β^U	0.0	0.0	0.0	0.0	1.000	1.000
Average heterozygosity		0.270	0.328	0.120	0.268	0.131	0.000

additional allele Tox^{U} at the low frequency of 0.092. The Akazai population of *P. maackii* also had the common allele Tox^{S} and another additional allele Tox^{F}. There are two alleles, Tox^{T} and Tox^{V}, in the population of *L. japonica*. The Fujimicho population of *L. puziloi* had no variability and was fixed with Tox^{T}. An example of the zymogram is shown in Figure 3. As shown in the figure, a heterozygote has only two distinguishable bands and no additional hybrid bands. Thus, the enzyme is a monomer in protein structure. Allelic frequencies are shown in Table 3.

Acid phosphatase (*Acph*): Enzyme assay was done by polyacrylamide gel electrophoresis. High variability and high allelic divergence was observed in this locus. Every allele had a mobility toward the anode. In the Akazai population of *P. bianor*, three alleles $Acph^{F}$, $Acph^{M}$ and $Acph^{S}$ were segregated at almost the same frequecies of 0.382, 0.284 and 0.333, respectively. Similarly, in the Ishigaki population of the species, there were also three alleles, $Acph^{K}$, $Acph^{P}$ and $Acph^{R}$, segregating at the approximately same frerquencies of 0.283, 0.317 and 0.400, but each of them had different mobility from those of the Akazai population. On the other hand, in the Nago population of *P. okinawensis*, there was no variability in Acph and it was fixed with the allele $Acph^{Q}$ which had its own mobility. Each allele was endemic to its own population. Thus, this may be useful as a good diagnostic characteristic which enables us to distinguish separate species one from another. The *P. maackii* population contained another two alleles $Acph^{L}$ and $Acph^{O}$. Those of *L. japonica* and *L. puziloi* were fixed with another two alleles, $Acph^{E}$ and $Acph^{D}$, respectively. An example of the

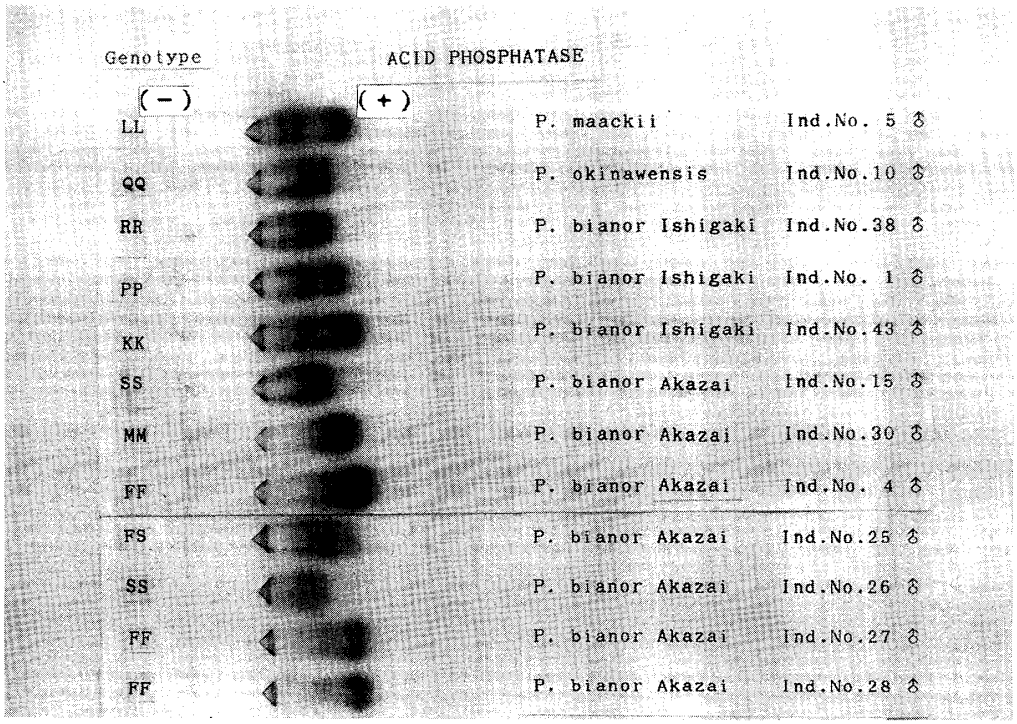

Fig. 4. An example of the zymogram of acid phosphatase (Acph) in the subspecies of *Papilio bianor* complex. Isozymes of Acph are stained in a brown color. Other description is same as in Figures 1 and 2.

Acph zymogram is represented in Figure 4. The heterozygote carried an additional hybrid band to its original two bands. Therefore, the enzyme must be a dimer in subunit structure. The situation is easily recognized in a heterozygote between alleles $Acph^F$ and $Acph^S$ in Figure 4. Allelic frequencies are summarized in Table 3.

Esterase-α (*Est-α*): The isozyme patterns were analyzed by agarose gel electrophoresis. At the enzyme locus, intrapopulational or intraspecific variation was not observed at all. Every population was uniform and fixed with a single specific allele. The Akazai and Ishigaki populations of *P. bianor* were fixed with the common allele Est-α^F. The Nago population of *P. okinawensis* and the Akazai population of *P. maackii* were fixed with another common allele Est-α^S. Both of the *Luehdorfia* populations were fixed with the other common allele Est-α^T. An example of the Est-α zymogram is shown in Figure 5. In the figure, esterase-α is one of substrate-nonspecific enzymes so that another esterase is also stained on the same gel plate. The number of subunits of the enzyme protein was unable to be determined because of the non-existence of the allelic heterozygote.

Esterase-β (*Est-β*): The isozyme patterns were analyzed by agarose gel electrophoresis. A total of four alleles, Est-β^F, Est-β^K, Est-β^M and Est-β^S, were segregated in the Akazai population of *P. bianor*. Their frequencies were 0.049, 0.010, 0.892 and 0.049, respectively, in this order. In the Ishigaki population of this species, two alleles Est-β^L and Est-β^R were segregated at almost even frequencies of 0.558 and 0.442. The polymorphism did not overlap with that of the Akazai population. On the

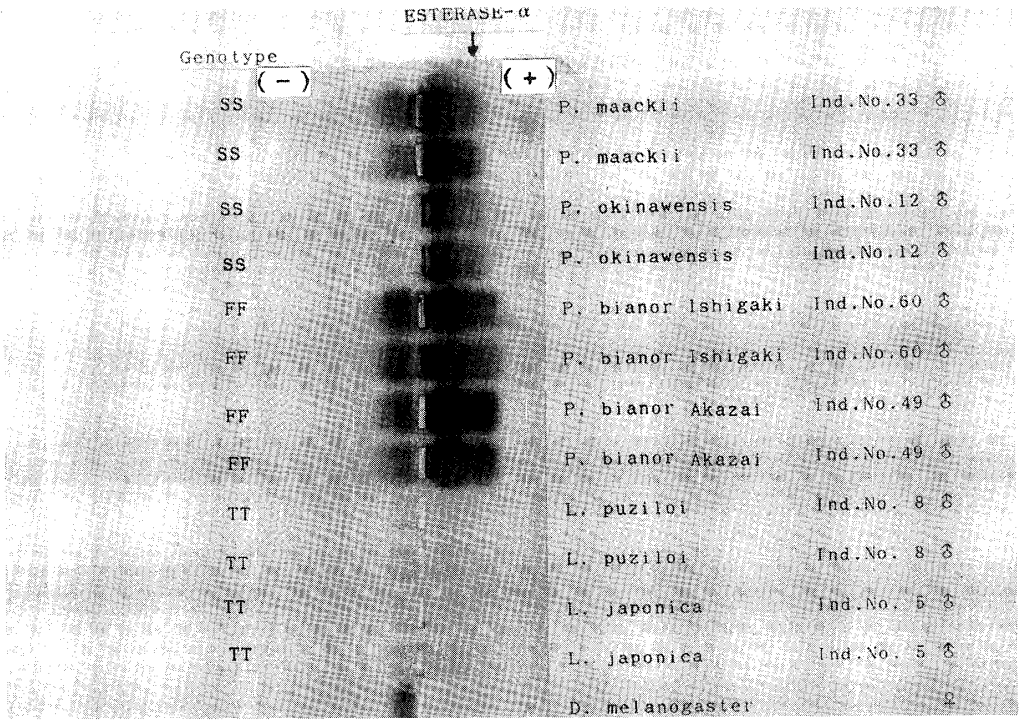

Fig. 5. An example of the zymogram of esterase-α (Est-α) in the subspecies of *Papilio bianor* complex. Isozymes of Est-α are stained in a deep brown color. D. melanogaster means the control isozymes of a fruit fly.

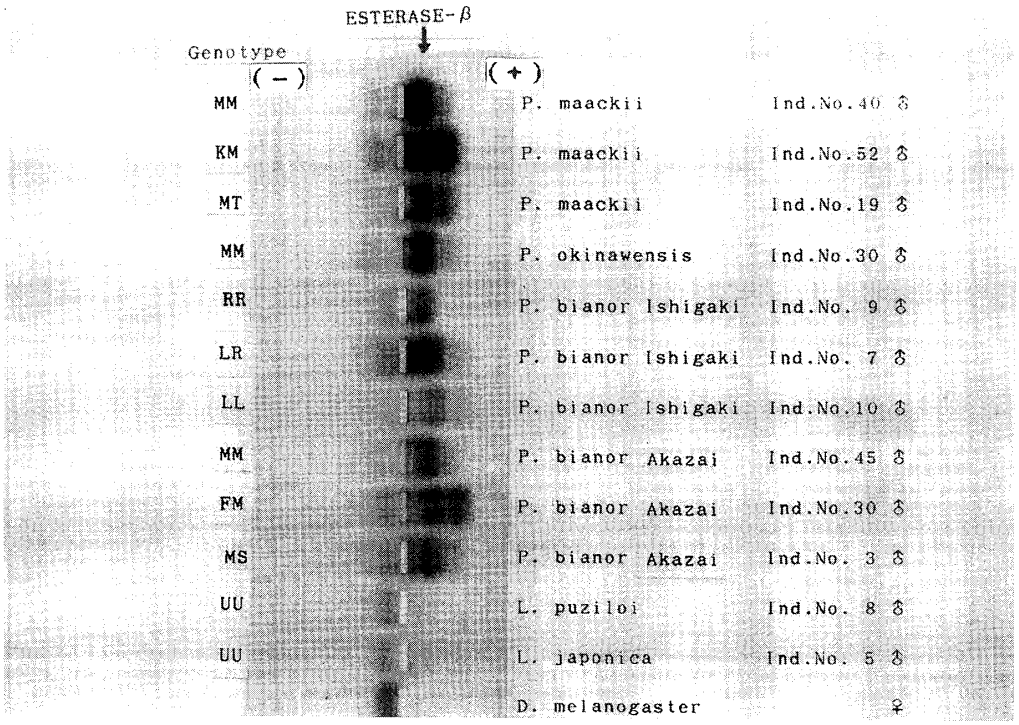

Fig. 6. An example of the zymogram of esterase-β (Est-β) in the subspecies of *Papilio bianor* complex. Isozymes of Est-β are stained in a red color. Other description is same as in Figures 1 and 2.

other hand, there was no Est-β polymorphism and fixation with Est-β^M in the Nago population of *P. okinawensis*. This allele was also the major one in the Akazai population of *P. bianor*. This was also the case in the *P. maackii* population, in which other two alleles, Est-β^K and Est-β^T, were segregated at low frequencies. In both of the *Luehdorfia* populations, the common allele Est-β^U was shared and fixed. An example of the zymogram is shown in Figure 6. The allelic heterozygote shows only both parental bands and indicates a structural monomer of the protein. These allelic frequencies are summarized in Table 3.

All the above zymographs at 6 isozyme loci are schematically shown in Figure 7. Relative mobilities of the isozymes are represented either in cathode direction or in anode direction from the origin.

2. Average heterozygosity at isozyme loci

Heterozygosity at a single locus (H) is defined by $1-\sum x_i^2$, where x_i is the gene frequency of the i-th allele. The average heterozygosity ($\bar{H}$) is obtained by an expansion of the formula into multiple loci.

$$\bar{H}=\frac{\sum_r(1-\sum_i x_i^2)}{r}$$

In this formula, r stands for the number of loci. The quantity is a good indicator of the magnitude of genetic variability, and is proportional to the effective size of the

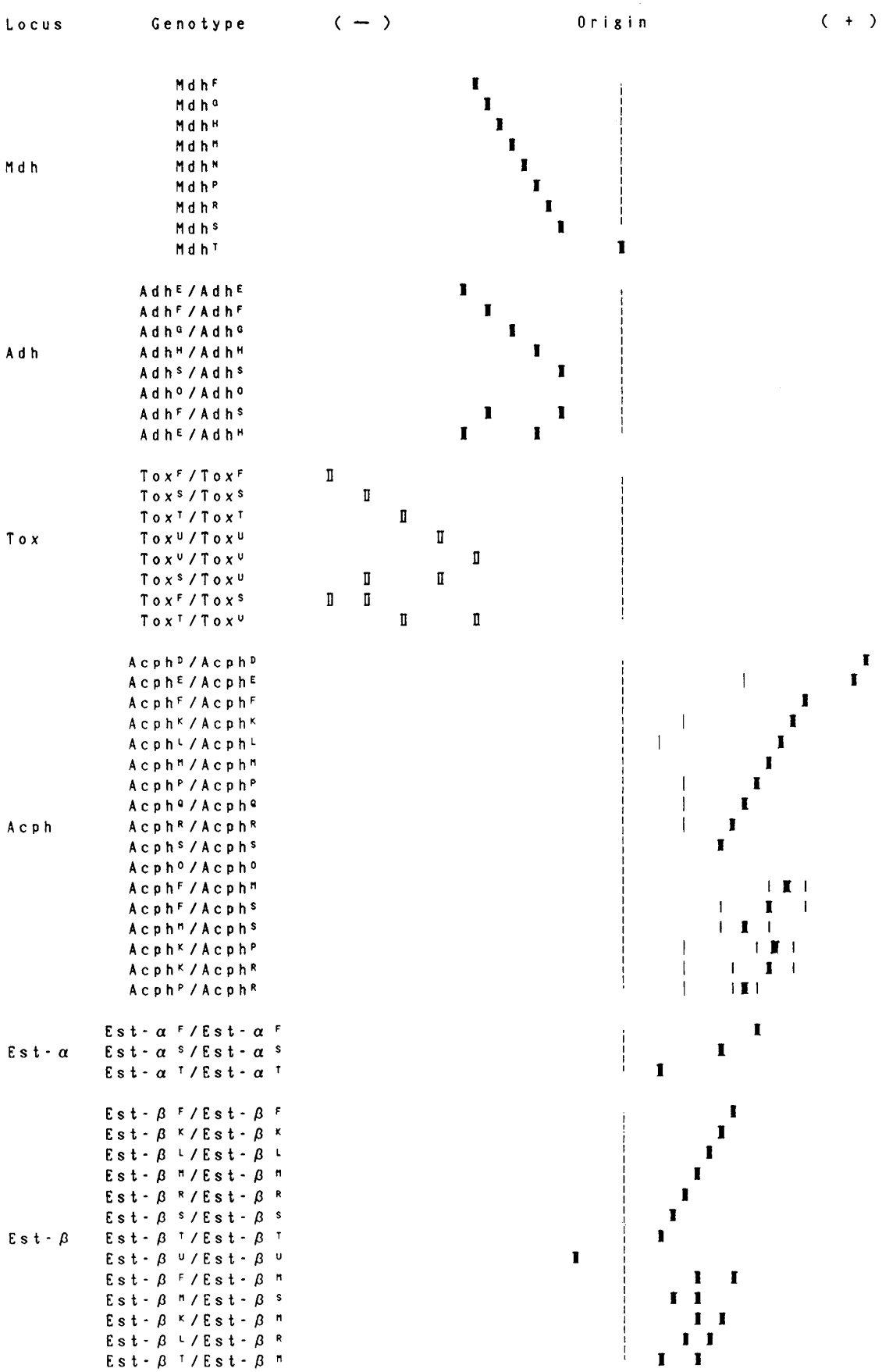

Fig. 7. Summary of schematic representation of the isozymes at 6 loci examined.

population. The average heterozygosity of the Akazai population of *P. bianor* was 0.270, that of the Ishigaki population of this species was 0.328, and that of the *P. okinawensis* population was 0.120. These values are not so small such as in a marginal population. In the population of its related species of *P. maackii*, the average heterozygosity was 0.268. For unrelated species, the average heterozygosity was 0.131 in the Nishiwaki population of *L. japonica*, and no heterozygosity was observed in *L. puziloi* population. This homozygosity seems to be due to the marginal location of the Fujimicho, Nagano, population for the distribution range of *L. puziloi*. Comparison of these $\bar{H}$ values is shown in Table 3.

3. Genetic distances between the subspecies of *P. bianor* complex

Using the above difference in gene frequencies, we can evaluate magnitude of genetic differentiation between the subspecies of *P. bianor* complex. Genetic similarity (I) and genetic distance (D) are calculated as foIlows (NEI, 1972).

$$J_x=\sum_j\sum_i\frac{(x_{ij})^2}{r}$$

$$J_y=\sum_j\sum_i\frac{(y_{ij})^2}{r}$$

$$J_{xy}=\sum_j\sum_i\frac{(x_{ij}\cdot y_{ij})}{r}$$

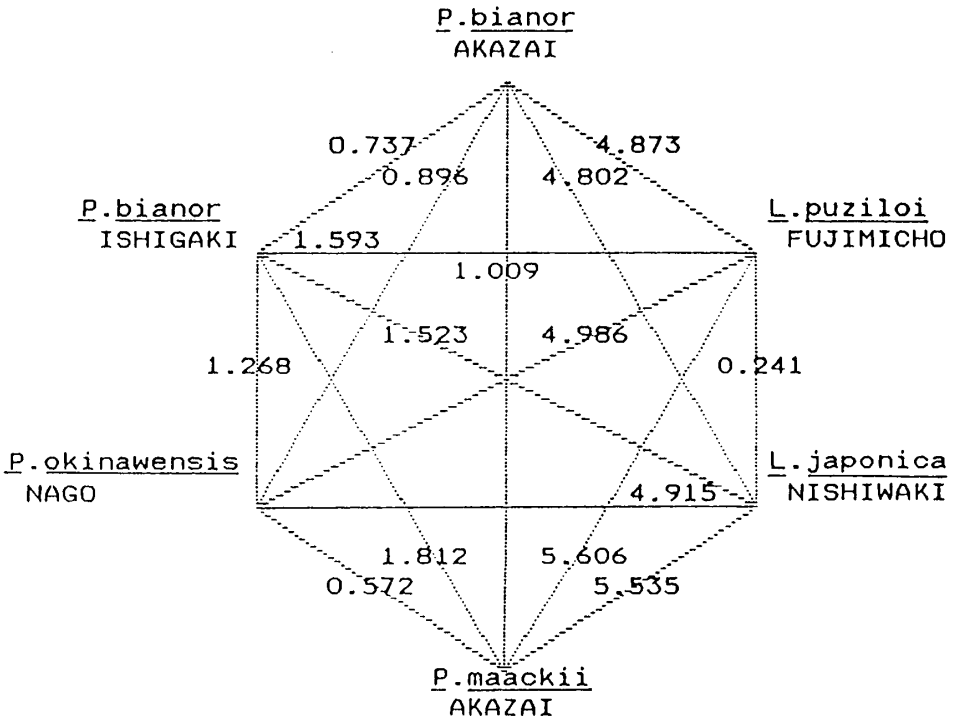

Fig. 8. The genetic distances between the subspecies of *Papilio bianor* complex, its related species of *P. maackii*, and its unrelated species of *Luehdorfia japonica* and *L. puziloi*. *P. bianor* from Akazai represents the subspecies of *P. b. dehaanii*. *P. bianor* from Ishigaki is synonymous with the subspecies of *P. b. okinawensis*. *P. okinawensis* is synonymous with the subspecies of *P. b. ryukyuensis*.

$$I = \frac{J_{xy}}{\sqrt{J_x} \cdot \sqrt{J_y}}$$
$$D = -\log_e I$$

In the formulae, x_{ij} and y_{ij} mean frequencies of i-th allele of j-th locus in population x and population y, respectively. r stands for number of loci examined.

The Genetic distance between the Akazai and Ishigaki populations of *P. bianor* was 0.737. The distance between the Akazai population of *P. bianor* and the Nago population of *P. okinawensis* was 0.896. The distance between the Ishigaki population of *P. bianor* and the Nago population of *P. okinawensis* was 1.268. All of these values are significantly greater than the described values of subspecies which range from 0.028 to 0.201, and are on the same level as that of described full species which range from 0.18 to 2.54 with an estimated mean value of 1.0 in insects. This mean value holds good also in all described species of animals, plants, fungi and bacteria (a review of NEI, 1975). Estimation of genetic distance between another separate species of Papilionidae butterflies was also tried in the control experiment. The distance between the Akazai populations of *P. bianor* and *P. maackii* was 1.009, and the value between *L. japonica* and *L. puziloi* was 0.241. These are in accordance with the above values, and are within the range for full species. The estimated genetic distances between the three subspecies of *P. bianor* complex in question seem to be genetically differentiated in the order of species. The genetic distances between all possible combinations of the subspecies and the species are illustrated in Figure 8. The genetic distance between the genera of *Papilio* and *Luehdorfia* was estimated to be 4.229.

Discussions

1. The locus of malate dehydrogenase is possibly sex-linked in Papilionidae butterflies

High genetic variability was observed at the Mdh locus in *Papilio* species examined in the present experiment. The average heterozygosity of the locus was 0.482. Thus, if we assume that the locus is on one of the autosomes, about a half of the number of examined individuals would be expected to show a heterozygotic zymo-graph. A total of 264 males were examined, however, none of them showed this type of zymograph but a hemizygotic one. A simple explanation is that the locus is on one of sex chromosomes. If so, then we can further expect a heterozygotic zymograph to be in the female. A total 36 females were examined in the same way, but none of them showed heterozygosity, contrary to an expectation. The female also showed hemizygotic zymographs as seen in Figure 9. These results taken together mean that in both sexes, the enzyme Mdh is produced from one of the genes on alternative homogous chromosomes. This type of gene expression is seen only in sex-linked loci. On the other hand, the karyotype of butterflies has been thuoght to be heterokryotypic in females (ZW typed) based on the low frequency of females among intra- or interspecific hybridization (e.g. AE, 1971). Thus, a possible explanation is that the Mdh

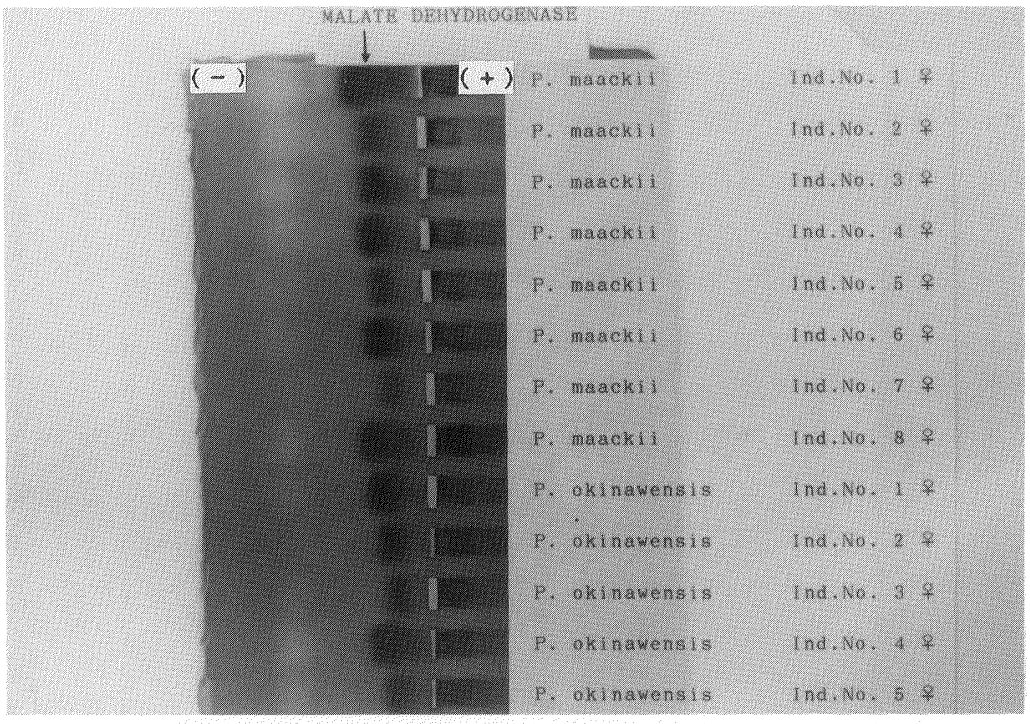

Fig. 9. The zymogram of malate dehydrogenase (Mdh) in the female butterflies of two species of *Papilio*. Isozymes of Mdh are stained in a deep blue color. There is no heterozygous zymograph formed by heterozygosis. All of them show a hemizygotic pattern.

locus is located on the Z chromosome in *Papilio* butterflies, and a mechanism of dosage compensation is involved in its expression. Further experiment is necessary to confirm this point.

2. Species diversity of *P. bianor* complex in island populations

Many investigators have continuously noticed that there is an extensive variation in the morphological characteristics in local populations of *P. bianor*. Color tone is brighter in nothern populations and is darker in southern populations. Some indices of wing traits are variable from population to population (IZUMI *et al.*, 1984). The shape of male genitalia is highly variable and is unique to some local isolates (KAWAZOÉ and WAKABAYASHI, 1976; ICHINOSE and HORIUCHI, 1985). Interpopulational hybridization has been carried out mainly by AE (1971 and 1985), HAMA (1976) and WAKI (1981), disclosing an unbalanced sex ratio in F_1 progeny and female sterility in some cases. From these observation, *P. bianor* in Japan is subdivided into five subspecies, *Papilio* (*Achillides*) *bianor dehaanii*, *P. b. amamiensis*, *P. b. tokaraensis*, *P. b. ryukyuensis*, and *P. b. okinawensis* (FUJIOKA, 1975). However, gene flow between the subspecies in nature is still unknown.

In this experiment, this possibility was directly examined through an analysis of isozyme genes. An enzyme is a direct product of a gene. Furthermore, a limited number of individuals in a population have a greater effect than the preferential pressure of natural selection on isozyme molecules, and make them selectively nearly

neutral. Isozyme analysis is therefore suitable for this kind of experiment. At both of the Adh and Acph loci, unique alleles were segregated independently between the populations of *P. b. dehaanii*, *P. b. ryukyuensis* and *P. b. okinawensis* as seen in Table 3. There was no indication of gene flow between the populations. On the other hand, migration of butterflies between islands has been observed frequentlly (e.g. AZUMA and MINATO, 1983). Sterility of F_1 hybrid females from a cross between *P. b. ryukyuensis* and *P. b. dehaanii* was reported by HAMA (1976). This is also the case in a cross between *P. b. okinawensis* and *P. b. dehaanii* (WAKI, 1981). Reproductive isolation between the subspecies seems to be perfect in nature.

Genetic divergence between the subspecies was also surveyed quantitatively in terms of the genetic distance, and the results are given in Figure 8. The genetic distances in any combinations between the subspecies were greater than 0.7. The magnitude was of the same order as that between *P. b. dehaanii* and *P. maackii tutanus*, or was more than that between *L. japonica* and *L. puziloi*. This magnitude is

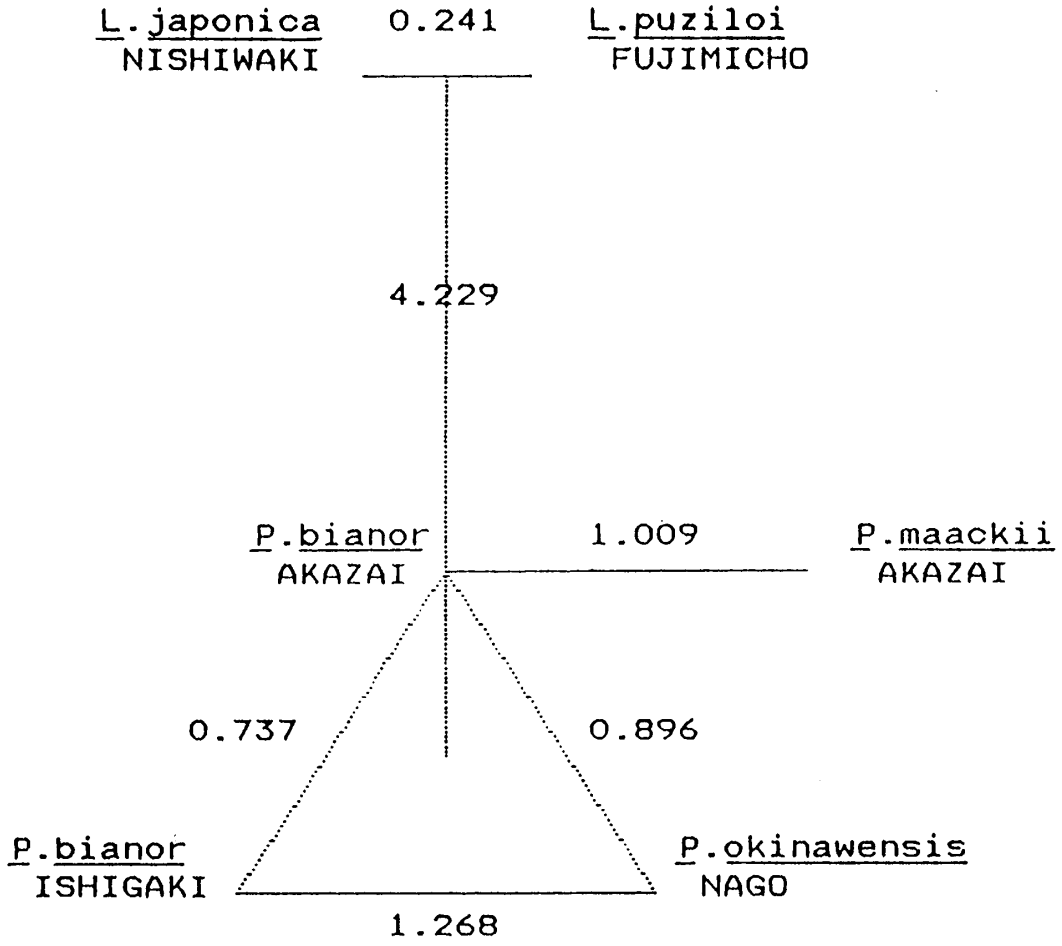

Fig. 10. The genetic distances between the subspecies of *Papilio bianor* complex (triangle), its related species of *P. maackii*, and its unrelated species of *Luehdorfia japonica* and *L. puziloi*. *P. bianor* from Akazai represents the subspecies of *P. b. dehaanii*. *P. bianor* from Ishigaki is synonymous with the subspecies of *P. b. okinawen--sis.*
P. okinawensis is synonymous with the subspecies of *P. b. ryukyuensis*.

also significantly greater than the mean value of genetic distances between subspecies examined so far, in the order of 0.1, and does not fall within the range of those of conspecific local populations, in the order of 0.01 (a review of NEI, 1975). Recent work by MATSUOKA *et al.* (1984) calculated the genetic distance between a pair of morphologically very similar species of *Neope goschkevitschii* and *N. niponica* to be 0.753. The summary of the present results is shown in Figure 10.

Adaptation of a subspecies to its own local habitat is possibly estimated through the amount of average heterozygosity ($\bar{H}$). The value of $\bar{H}$ of *P. b. ryukyuensis* was 0.120, and that of *P. b. okinawensis* was 0.328, as seen in Table 2. Both of these did not so deviate too much from that of *P. b. dehaanii*, 0.270. Thus, each subspecies population has comparable genetic variability, and it is further suggested that it had a long time, to build up to the amount of present genetic variabililty after the foundation of the ancestral population.

The above observations are strongly suggestive of speciation in progress in *Papilio bianor* complex, especially in island populations. Some of present subspecies of *P. bianor* may be reclassified into new independent species. Recently, KAWAZOÉ and WAKABAYASHI (1976) described subspecies of *P. b. ryukyuensis* as a new species of *P. okinawensis* based on morphological differences. Similarly, ICHINOSE and HORIUCHI (1985) described subspecies of *P. b. okinawensis* as a new separate species based on genetical analysis. The present experimental results support these author's conclusions and give additional experimental evidence. Similar speciation is thought to be in progress also in other butterflies in Japanese southwest island populations.

Acknowledgements

The authors express their thanks to Miss Megumi KASAHARA for her kind help in the calculations and in making graphics by computer. They also thanks to Dr. Kei-ichi OMOTO for his valuable comments toward the manuscript, to Mr. Albert J. CHICK for correcting English and to Messrs. Morishige SHIMABUKURO and Ken-ichi WATANABE for their kind help in the collection of the present materials in Okinawa. One of the authors (H.K.) is deeply indebted to Dr. Nobutaka ITO for his encouragement throughout this experiment.

References

AE, S. A., 1971. A further study of interspecific hybrids in *Papilio*. *Spec. Bull. Lep. Soc. Japan*, **5**: 89–114.

——— 1985. Studies on *Papilio bianor* complex in Japan and Formosa through breeding and hybridization experiments. *Tyô to Ga*, **36**: 206. (In Japanese.)

AZUMA, S. & K. MINATO, 1983. Butterflies of Ryukyus. 126 pp. Shinseitoshoshuppan, Naha. (In Japanese.)

FUJIOKA, T., 1975. Butterflies of Japan. 329 pp. Kodansha, Tokyo. (In Japanese.)

HAMA, S., 1976. On the hybridization between the two subspecies of *Papilio bianor* from Honshu and Okinawa Is. *MDK NEWS*, **26**: 12–16. (In Japanese.)

ICHINOBE, T. & C. HORIUCHI, 1985. Allopatric speciation of Yaeyama insular isolates in the *Papilio bianor* complex of Japan (Lepidoptera, Papilionidae). *Kontyû, Tokyo,* **53**: 233－244.

IZUMI, K., K. KANO & K. SASAKI, 1984. Studies on the geographic variation of wing shape in *Papilio bianor* by principal component analysis. *Tyô to Ga*, **34**: 155－162. (In Japanese.)

KAWAZOÉ, A. & M. WAKABAYASHI, 1976. Colored illustrations of the butterflies of Japan. 442 pp. Hoikusha, Osaka. (In Japanese.)

KIMURA, M., 1982. The neutral theory as a basis for understanding the mechanism of evolution and variation at the molecular level. *In* M. KIMURA, *Molecular evolution, protein polymorphism and the neutral theory*, 363 pp. Japan Scientific Society Press, Tokyo, and Springer-Verlag, Berlin.

KOJIMA, K., J. GILLESPIE & Y. N. TOBARI, 1970. A profile of Drosophila species' enzymes assayed by electrophoresis. I. Number of alleles, heterozygosities, and linkage disequilibrium in glucose-metabolizing systems and some other enzymes. *Biochem. Genet.*, **4**: 627-637.

MATSUOKA, N., Y. CHIBA & K. SAITOH, 1984. Biochemical evidence for the genetic differentiation between two morphologically very similar species of *Neope* (Lepidoptera, Satyridae) from Japan. *Proc. Japan Acad.*, **60**: 245－248.

NEI, M., 1972. Genetic distance between populations. *Amer. Nat.*, **106**: 283－292.

――― 1975. Molecular population genetics and evolution. 288 pp. North-Holland Publishing Co., Amsterdam.

STONE, W. S., F. M. JOHNSON, K. KOJIMA & M. R. WHEELER, 1968. Isozyme variation in island populations of *Drosophila*. I. An analysis of a species of the *nasuta* complex in Samoa and Fiji. *Univ. Texas Publ.*, **6818**: 157－170.

WAKI, I., 1981. On the experimental results of hybridization between species of *Achillides* group. *Rhopalocerists' Mag.* [Cho Cho], **4**: 5－10. (In Japanese.)

摘　　要

カラスアゲハとその近縁種間のアイソザイム遺伝子頻度より解析した分化程度

（小南裕彦・山口　修・住吉　薫）

カラスアゲハ (*P. bianor* complex) は，中国大陸・朝鮮・台湾・沖縄諸島・日本本土に広く分布し，そのはねの模様・色合いも各分布地で微妙に変化している．この傾向は特に，南西諸島のような海で隔たった隔離小集団では強いが，形態学的には現在7亜種に分類されることが多い．川副・若林(1975)では，このうち沖縄諸島産を *P. okinawensis* とし，一ノ瀬・堀内(1985)は，八重山諸島産のものを別種としている．

しかし，これら亜種間でのお互いの交配実験も多数試みられてはいるが，F_2までの飼育が困難でもあり，種(species)レベルまでの分化が進んでいるのか，あるいはさらに多数の別種のレベルまで分化しているのか，実験的な確証は得られていない．

本研究では，これらの関係の一端を調べるため，1986年にカラスアゲハの本州産，沖縄本島産，石垣島産の雄を各51，52，60匹採集した．

腹部を解剖し，精巣および消化器官に含まれる酵素のうち，リンゴ酸脱水素酵素 (Mdh)，アルコール脱水素酵素 (Adh)，テトラゾリウム酸化酵素 (Tox)，酸性燐酸化酵素 (Acph)，α-エステル化酵素 (Est-α)，β-エステル化酵素 (Est-β) の6遺伝子座につき，その対立遺伝子頻度を電気泳動法で調査した．同一の移動パターンおよび特異的移動パターンを持つものの割合より，NEI(1972)の式を用いて，互いの遺伝的距離を推定した．遺伝的距離は2集団間でのすべての同一移動度を持つアイソザイム遺伝子の頻度の相関の逆数として表される．

本州産と沖縄本島産のものでの遺伝的距離は，0.896であり，本州産と石垣島産，沖縄本島産と石垣島産のそれは，0.737と1.268であった．これらの値は，対照区にとったカラスアゲハとミヤマカラスアゲハ (*P.*

maackii) の 1.009, および, ギフチョウ (*L. japonica*) とヒメギフチョウ (*L. puziloi*) の 0.241 と同程度のオーダーに分化していることを示している. これらの値は, NEI の総論 (1975) の他の動植物で得られている種間の値 ; 0.18－2.54 の範囲内に入っている.

同一種内の他方品種間では, 0.01 のオーダー, 亜種間では, 0.1 のオーダー, 属間では, 1.10 以上のオーダーからみても種のレベルに最も近い.

また, Adh および Acph では, 3 産地で共に共通の対立遺伝子は全く存在しておらず, 野外では遺伝子の交流が無いことを示すとともに, 固有の対立遺伝子に固定していることを示している.

これらのことは, 南西諸島のカラスアゲハは, 遺伝的には別種としても良い程度に分化を起こしていることを示唆している.